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Mechanism of JmJc-containing protein Hairless in the regulation of vitamin D receptor function

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ABSTRACT

The JmJc-domain-containing protein Hairless (HR) and the vitamin D receptor (VDR) play a critical role in the maintenance of hair growth. Mutations in HR or VDR cause alopecia in humans and mice. Here we show that HR interacts with VDR and induces VDR relocalization in the nuclei. HR associates and colocalizes with nuclear receptor co-repressor (N-CoR) which is localized to subnuclear structures termed matrix-associated deacetylase (MAD) bodies. It is found that the HR mutants (C622G, N970S, D1012N, V1136D), associated with alopecia universalis congenita (AUC) or atrichia with papular lesions (APL), exhibit an abnormal subcellular distribution in addition to the impaired co-repressor activity with VDR. Studies on deletion mutants of HR indicate that the JmJc domain contributes to the co-repressor activity of HR. Our work provides new clues and evidence for the understanding on the role of HR in hair growth.

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1. Introduction

Histone methylation regulated by histone methyltransferases (KMTs) and histone lysine demethylases (KDMs) play important roles in gene expression and cellular differentiation [1,2]. A number of JmJc-domain-containing proteins have been identified as KDMs. Hairless (HR) protein shares particular similarity with the JHDM2 family proteins (JHDM2A, B, and C) which contain a zinc finger and the JmJc domain [1]. An interesting feature that is shared among JHDM2 proteins is their involvement in nuclear hormone receptor (NR)-mediated functions. JHDM2A, which has been identified as a histone H3K9 demethylase, contributes to AR-mediated gene activation [3]. The function of JmJc domain in HR is unclear.

HR is a nuclear protein that is expressed mainly in skin and brain [4,5]. HR has been shown to act as a co-repressor of multiple nuclear receptors, including thyroid hormone receptor (TR), the retinoic acid receptor-related orphan receptors (RORs) and the vitamin D receptor (VDR) [6–9]. Both the mammalian HR and VDR are important in hair growth [10–13]. Mutations in either *hr* or *vdr* gene result in congenital hair loss in both mice and human beings [14–16]. Moreover, the alopecia caused by mutations in the *vdr* gene resembles that occurred in the *hr* gene [14,16]. However, the molecular mechanisms by which HR (and VDR) exerts its effects on the skin and on hair growth are still being elucidated.

Here we show that HR interacts with VDR in the nuclei where it colocalizes with N-CoR. The HR pathogenic mutants lacking corepressor activity exhibit abnormal subcellular distribution and altered nuclear retention.

2. Results

2.1. HR represses VDR-mediated transactivation

To examine the effects of human HR on VDR mediated gene expression, calcitriol-responsive reporter constructs (CYP24A1 promoter, known as targets of VDR, Fig. S1A) and a VDR expression plasmid were co-transfected into the HaCaT (or HeLa) cells with or without HR expression plasmid. As expected, CYP24A1 promoter activity was significantly induced by calcitriol. It is found that HR blocked calcitriol induced promoter activity (Fig. 1A, S1B and C). The results are consistent with previous reports that HR suppressed both basal and liganded VDR transcriptional activity [17,18].

It has been hypothesized that the repression by HR is mediated through the direct interaction of HR and VDR. Co-immunoprecipitated assay showed that endogenous VDR was in complex with Flag-HR (Fig. 1B). To address whether the interaction was taking place in the nucleus or in the cytoplasm, we examined the subcellular distribution of HR and VDR. GFP tagged HR showed a discrete dot pattern in HaCaT or HEK293T cells (Fig. 1C, S2A and S2B). Cherry-VDR alone is distributed in both the cytoplasm and nucleus (Fig. S2C). When HR and VDR were co-expressed in HEK293T cells, the cherry-VDR co-localized with HR at nuclear speckles (Fig. 1D). Interestingly, calcitriol did not show any effect on this co-localization (Fig. S2D).

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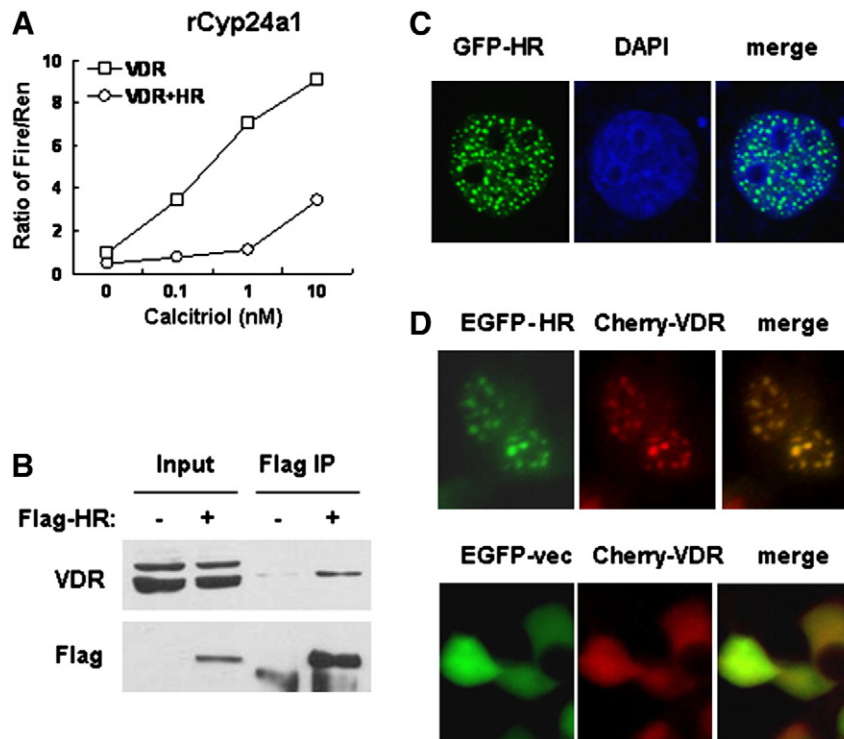


Fig. 1. HR represses VDR-mediated transactivation. (A) CYP24A1-Luc and TK-RL were co-transfected into HaCaT cells together with an expression vector for HR or expression vector alone. Luciferase activity was measured with graded concentrations of calcitriol. (B) Protein extracts prepared from HeLa cells transfected with an expression vector for Flag-HR or expression vector alone were used for immunoprecipitation with anti-Flag M2 beads. Endogenous VDR is detected only when HR is expressed. (C) Subcellular distribution of GFP-HR in HaCaT cells. Nuclei were visualized by DAPI staining. Cells were observed under a laser confocal microscope. (D) GFP-HR and Cherry-VDR were transfected into 293 T cells individually or together. When the two proteins were co-expressed at proper levels, cherry-VDR colocalized to HR nuclear speckles.

2.2. HR associates and colocalizes with N-CoR

In a Flag-immunoprecipitation experiment with extracts from HeLa cells that expressed Flag-HR fusion protein, associated polypeptides were identified by mass spectrometry. These proteins include the followings: the N-CoR, the histone deacetylases HDAC3, and the histone methyltransferase (HMT) G9a (Fig. 2A). The specificity of the results was validated by co-immunoprecipitation (Fig. 2B, C). N-CoR, HDAC3, but not G9a was immunoprecipitated by Flag-HR. mSin3A, a member of N-CoR complex [19], was also detected by Western blotting analysis. So the complex composed of N-CoR, HDAC3 [20], and mSin3A may contribute to the corepressor function of HR.

It has been reported that N-CoR is localized to subnuclear structures termed matrix-associated deacetylase (MAD) bodies [8]. To determine whether HR locates to the same region with N-CoR, co-localization experiments were performed in HEK293T cells. It was shown that the pattern of the two proteins overlapped well (Fig. 2D). The data implies that HR and N-CoR may reside in a multiprotein complex which serves the transcriptional repression function.

We also observed that HR was located in regions in the nucleus distinct from HP1 α (Fig. 2D) which is reported to be enriched at heterochromatic foci [21]. Our findings suggest that HR is independent of HP1 α complex.

2.3. HR pathogenic mutants defective in corepressor activity are abnormally distributed

A number of different mutations in HR have been described in patients with APL/AUC [15]. These mutations occur primarily within the JmJc and zinc finger domain (Fig. 3A). To assess the impact of these mutations on HR co-repressor activity, four mutants (C622G, N970S, D1012N, V1136D) previously described as the molecular cause

of APL/AUC as well as three HR variants (C397Y, E591G, T1022A) were constructed by site-directed mutagenesis. The co-repressor activity of the mutant HRs on VDR transcription was then examined in HeLa and HaCaT cells. All of the HR mutants completely lost their co-repressor activity while the variants fully retained their repressive ability (Fig. 3B in HeLa cells, S3A, B in HaCaT cells and [17]).

To further explore biological differences of HR mutants, their subcellular distribution patterns were investigated. The four HR mutants and three variants tagged with Flag or GFP were expressed in HeLa or HEK293T cells, and examined by indirect immunofluorescence. To our surprise, all the pathogenic mutants showed an abnormal subcellular distribution. The mutant proteins were diffusely distributed in the nucleus and cytoplasm. No dot structures were observed in the nucleus (Fig. 3C, S4 and S5). In contrast, the pattern of the HR variants (C397Y, E591G, T1022A) was similar to that of the wild type HR (Fig. 3D).

2.4. HR mutants display an altered nuclear retention ability

During the course of localization study, we noted that native HR and HR variants adopted a fine punctate subnuclear expression pattern in 293 T cells, whereas the four HR mutants were widely distributed. This suggests that HR may associate with structural components within the nucleus and HR mutants lost this specialization. Biochemical fractionation and *in situ* extraction studies showed that proteins of pathogenic mutants described above easily leaked out of the nucleus under a detergent treatment in contrast to the polymorphic variants, indicating that the nuclear retention ability of the four HR mutants were impaired (Fig. 4A and B).

As described above, both the wild type HR and N-CoR were localized to the MAD body, however, there's a lack of co-localization between the HR pathogenic mutants and N-CoR (Fig. 4C). We speculate that the

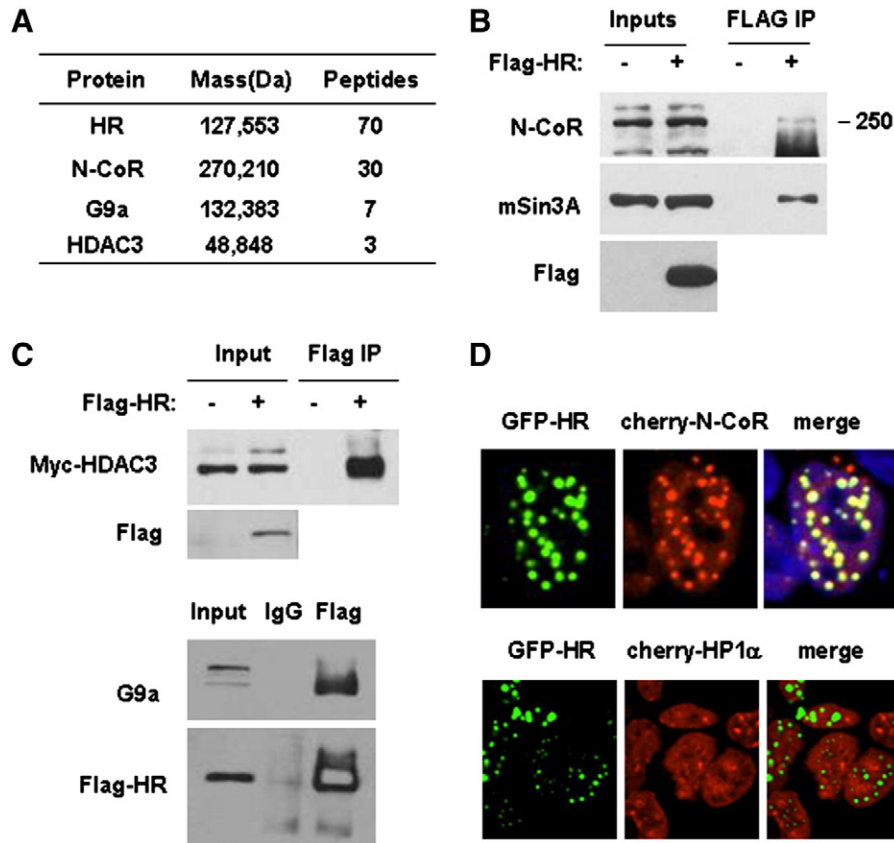


Fig. 2. HR associates and colocalizes with N-CoR. (A) Summary of the peptides identified by mass spectrometry from an anti-Flag purification of Flag-HR expressed in HeLa cells. Mass and observed peptides are indicated for each protein. (B) Endogenous N-CoR and mSin3A are associated with ectopically expressed Flag-HR. A Representative western blot, using antibodies to the indicated proteins, of lysates prepared from HeLa cells with expression of Flag-HR. (C) Flag-tagged HR and myc-HDAC3 were coexpressed in 293 T cells. Myc-HDAC3 was detected by Western blot when HR was immunoprecipitated by anti-Flag beads. No endogenous G9a was found associated with HR in 293 T cells. (D) HR colocalizes with N-CoR but not HP1α. 293 T cells were co-transfected with expression vectors for GFP-HR and Cherry-N-CoR. Both HR and N-CoR showed nuclear, dot pattern and the pattern of the two proteins overlapped well. GFP tagged HR and Cherry-HP1α localized in distinct regions in the nuclei. Cells were observed under a laser confocal microscope.

MAD body is essential for the co-repressor activity of HR and is critical for its role in maintaining the normal hair cycle in mammalian skin.

2.5. The JmjC domain is indispensable for the corepressor activity of HR

To investigate the role of JmjC domain of HR, we generated serial HR truncated mutants (Fig. 5A). While full-length HR (amino acids 1–1189) was located in the nuclei of transfected HEK293T cells, the mutant (1–994) without JmjC domain led to a peri-nuclear envelope pattern (Fig. 5C). Promoter activity assay showed that the mutant (1–994) retained only a little repression activity (Fig. 5B).

The mutant (454–1189), which removed the nuclear localization signal (NLS, [22]) and one repression domain while preserved the JmjC domain, showed a cytoplasmic localization pattern and decreased repression activity (Fig. 5B and C).

The mutants (1–730 and 1–463), lacking two repression domains in addition to the JmjC domain, were located in the nucleus again (Fig. 5C). The mutants (454–994 and 677–994) composed of only two repression domains showed a cytoplasmic localization (Fig. 5C). These four mutants were unable to deliver an efficient repression activity (Fig. 5B).

3. Discussion

It is known that mutations in HR cause congenital alopecia in humans and mice. Previous studies reveal that HR regulates the differentiation of epithelial stem cells thus the hair cycle [10]. HR

interacts with the VDR in the nuclei and has been shown to function as a corepressor of VDR. Most of the pathogenic mutations described in patients with APL/AUC impaired the corepressor activity of HR, which hints the importance of the corepressor activity of HR in normal hair growth. In addition, the pathogenic mutations were mainly identified in the JmjC domain of HR, suggesting a pivotal biological role of JmjC domain in normal hair growth.

It has been shown that HR colocalizes with components of MAD bodies such as SMRT and histone deacetylase HDAC5 [8]. We found that while HR colocalized with N-CoR, a homologous protein of SMRT, the HR pathogenic mutants (C622G, N970S, D1012N, V1136D) did not. These data raised a question on whether HR and N-CoR/SMRT reside in a large multiprotein complex and are functionally linked. However, knockdown of N-CoR or SMRT by shRNA in HeLa cells showed little influence on the repression activity of HR (data not shown).

HR is a nuclear protein with a JmjC domain in its C-terminal region. JmjC-domain-containing proteins play important roles in regulating gene expression and other processes within an epigenetic modification system. Although the HR protein lacks conserved coregulator binding sites found in histone demethylases, some results suggest that the JmjC domain in HR has a similar enzymatic activity with novel specificity [23]. Additionally, HR may function in a way similar to the protein Jarid2 which is devoid of detectable histone demethylase activity, but regulates gene expression by interacting with the PRC2 complex [24,25]. However, influence of HR on histone marks was not observed in the ChIP assay (Fig.S6).

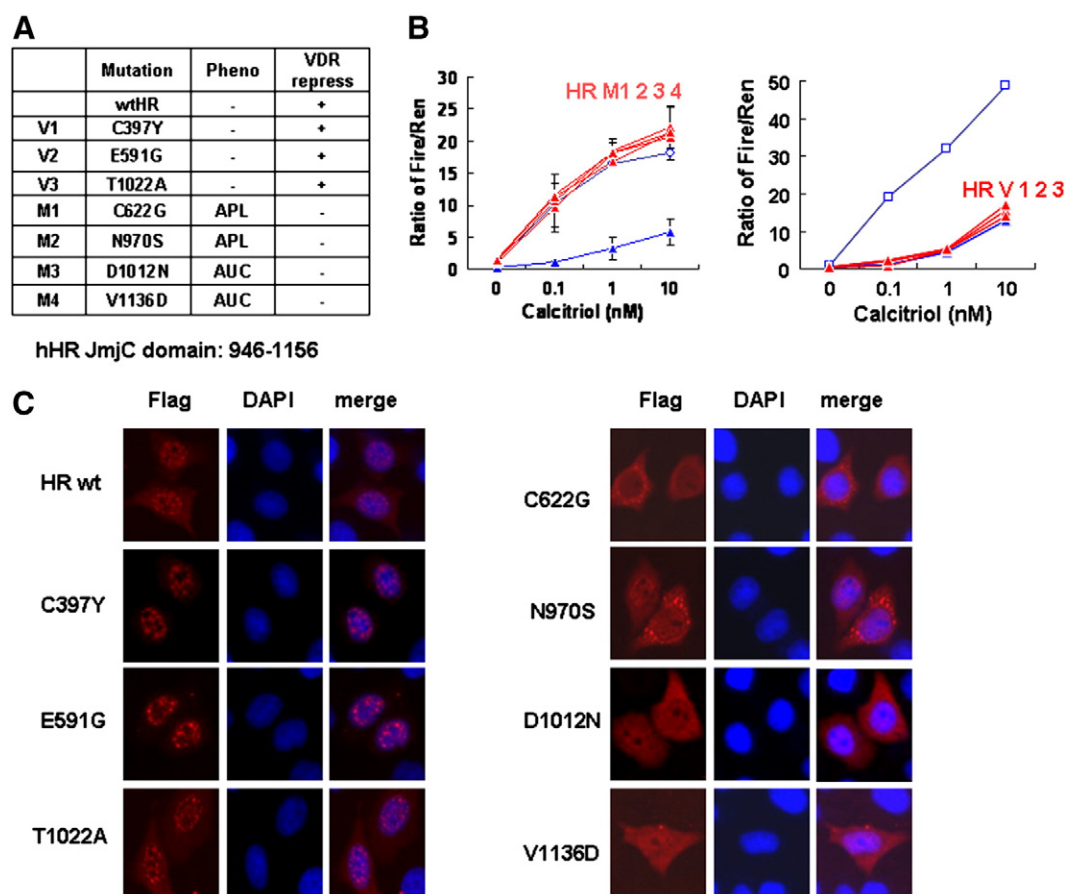


Fig. 3. Impaired corepressor activity and abnormal subcellular distribution of HR pathogenic mutants. (A) Summary of corepressor activities of HR pathogenic mutants tested in this study. Our results are consistent with previously published data (Wang et al. 2007). (B) HR pathogenic mutants lost the corepressor activity with VDR. HeLa cells transfected with CYP24A1-luc reporter and an expression vector for one of the four HR mutants (HRm1–4) were used for luciferase assay. HR variants (HRv1–3) served as control. (C) HR pathogenic mutants (C622G, N970S, D1012N, V1136D) were mislocalized in HeLa cells. HeLa cells were transfected with an expression vector for one of the Flag tagged HR mutants previously reported as the molecular cause of APL/AUC and detected by indirect immunofluorescence. HR variants (C397Y, E591G, T1022A) showed the same distribution pattern as the wild type.

The HR pathogenic mutants (C622G, N970S, D1012N, V1136D), to our great surprise, exhibited diffuse distribution in the cytoplasm and nucleus. They failed to be enriched at MAD bodies and had altered nuclear retention. We noticed that these mutations were mainly located in the hydrophobic region around the JmjC domain where one amino acid change may cause a protein conformation change. Previous studies show that although nuclear receptor interaction domains also reside in this region [6], mutants lacking suppression activity still retain interaction with VDR [17]. The distribution pattern of HR mutants was different from the one of WT HR when co-expressed with VDR (Fig.S7). We speculate that these mutants lost partners critical for activity such as HDAC1 [17], and nuclear components for MAD body targeting other than the VDR protein. The C622G mutation is in the putative zinc-finger domain [4], so the reduced corepressor activity and abnormal distribution of the mutant suggests that the amino acid in this site is critical for DNA interaction.

Both the mutant (1–994) lacking the JmjC domain while preserving the VDR binding region and repression domains, and the mutant (454–1189) lacking the NLS while saving the JmjC domain exhibited altered subcellular localization and decreased repression activity. It suggested that preserving integrity of JmjC domain and normal nuclear localization were essential for the biological function of HR. The mutants (454–1189, 454–994 and 677–994) did not colocalize with VDR or N-CoR in nucleus (data not shown), which may partially explain the situation.

In summary, we found that HR mutants causing alopecia carry a distribution different from the WT HR that was located with N-CoR in MAD bodies. Our results suggest that the JmjC domain integrity

and HR protein normal localization are required for the correct cycling of the hair follicle.

4. Materials and methods

4.1. Cell culture

HeLa, HEK293T and HaCaT cells were grown in Dulbecco's modified Eagle's medium containing 10% bovine growth serum (HyClone Laboratories, Inc.). Cells were incubated at 37 °C under a 5% CO₂ atmosphere.

4.2. Plasmids

Full-length human HR cDNA was subcloned from pOZ-HR (kindly provided by Dr. Pinchao Mei, Peking Union Medical College, Beijing) into pcDNA6/V5-FLAG and pEGFP-C1 vector. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). All clones were sequenced to confirm the presence of the mutation. YFP-C3-mN-CoR (AA962-2455) was a generous gift from Dr. Richard N. Day (Indiana University School of Medicine, Indianapolis). Fragments of the rat CYP24A1 promoter (–1399 to +76) and human CYP24A1 promoter (–812 to +44) were cloned by PCR from rat or human genomic DNA into pGL3-Basic vector (Promega).

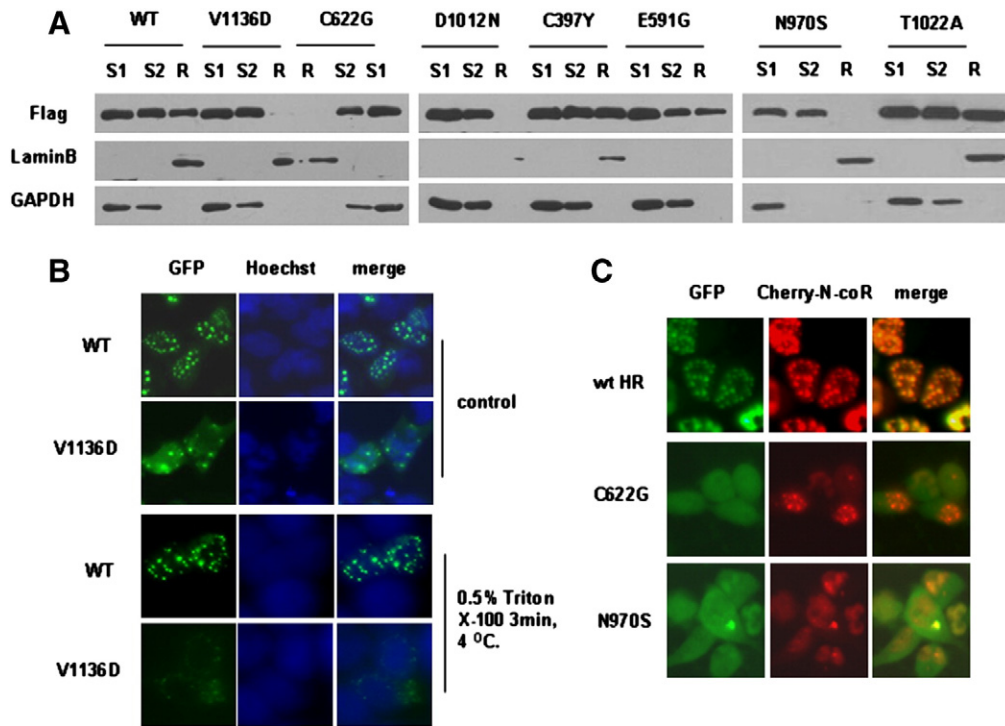


Fig. 4. HR pathogenic mutants exhibit altered nuclear retention. (A) 293 T cells transfected with HR mutant or variant were fractionated into detergent-soluble (S) and -resistant (R) fractions with swelling buffer (25 mM Hepes, pH7.8, 1.5 mM MgCl₂, 10 mM KCl, 0.1% Nonidet P-40). Cells were extracted twice in swelling buffer and the supernatants were saved as S1 and S2 fractions. The insoluble structure was extracted with RIPA buffer and used as the resistant (R) fraction. Extracts from each step were analyzed by Western blots. (B) *In situ* extraction of HR and HR V1136D mutants. 293 T cells transiently transfected with GFP tagged HR or HR mutant were subjected to Triton X-100 extraction (0.5% Triton X-100, 3 min, 4 °C) and observation without any fixation. WT HR was located in punctate subnuclear foci and these foci preserved during the extraction procedure. In contrast, the mutant HR failed to retain in the nucleus. (C) Wild type HR was colocalized with N-CoR in 293 T cells whereas HR C622G and N970S mutants were not. Cells were transfected with wt HR or HR mutants and observed 24 h later.

4.3. Indirect immunofluorescence

Cells were grown on poly-lysine coated glass coverslips and fixed in a freshly prepared mixture of methanol: acetone (1:1) for 1 min at room temperature. Cells were stained with mouse monoclonal Flag M2 antibody (Sigma) at a concentration of 10 µg/ml in TBS for 2 h at room temperature. Secondary TRITC-conjugated antibodies were

used at a dilution of 1:250. Coverslips were washed, stained with DAPI, mounted and analyzed by fluorescence microscopy.

4.4. Luciferase assay

HeLa cells were grown to 60–80% confluence in 24-well tissue culture plates. Cells were transfected with either 80 ng of WT VDR with or

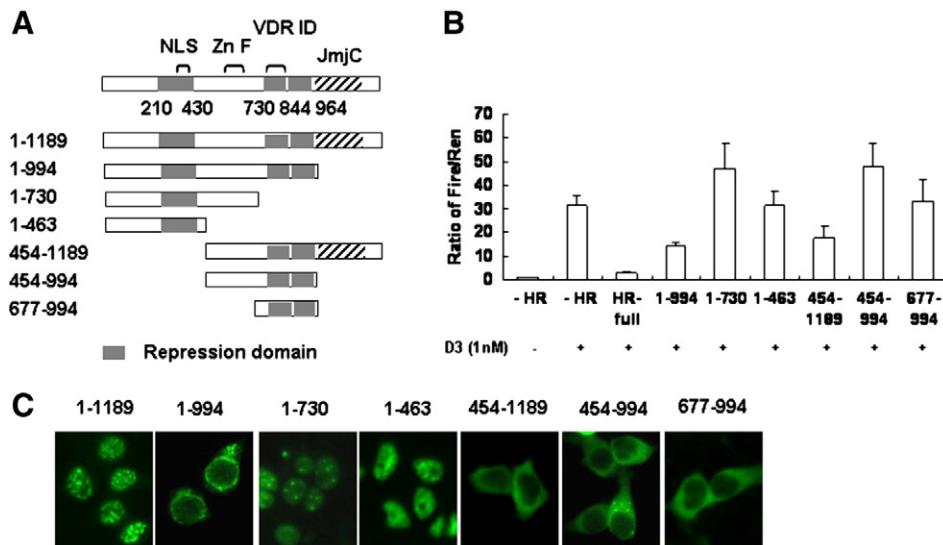


Fig. 5. Deletion of JmjC domain impairs the activity and distribution of HR. (A) Schematic representation of human HR truncation mutant strategy. NLS, 412–430; cysteine-rich domain, 567–692; VDR Interaction Domain, 730–844; JmjC domain, 946–1156. (B) Corepressor activities of HR truncation mutants with VDR in 293 T cells. (C) Subcellular localization of GFP tagged HR missing mutants. 293 T cells were transfected with expression vectors for the indicated HR-GFP fusion proteins. GFP-positive cells were photographed 24 h after transfection.

without 400 ng of human WT HR or HR mutant expression plasmids and 400 ng of CYP24A1 promoter luciferase plasmid. pRL-TK Renilla luciferase plasmid served as an internal control for transfection efficiency. After a 24-h transfection, the cells were incubated 14–20 h in Dulbecco's modified Eagle's medium containing 1% bovine growth serum with vehicle or calcitriol. The transfected cells were lysed in 100 μ l of Passive Lysis Buffer (Promega) and assayed luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and GloMax 96 Microplate Luminometer (Promega).

4.5. Western blotting and immunoprecipitation

HEK293T cells were transfected using VigoFect transfection reagent and Lipofectamine 2000 (Invitrogen) was used to transfect HeLa cells. Cells were lysed in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, and a protease inhibitor mixture. For immunoprecipitation, cell lysates were incubated with Flag M2 beads (Sigma) 4–8 h at 4 °C followed by extensive wash with the lysis buffer. The immunoprecipitates were analyzed by SDS-PAGE.

Antibodies for Flag (F3165, Sigma), GFP (sc-8334, Santa Cruz), GAPDH (MAB374, Millipore), VDR (sc-1008X, Santa Cruz), mSin3A (sc-994, Santa Cruz), N-CoR (gift from Dr. Jiemin Wong, East China Normal University, Shanghai) and G9a (3306, cell signaling) were used.

4.6. Cell fractionation

293 T cells transfected with different HR mutants or variants were fractionated into detergent-soluble and -resistant fractions with swelling buffer (25 mM Hepes, pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, 0.1% Nonidet P-40). 2–5 $\times 10^7$ cells were extracted twice for 10 min on ice with swelling buffer, the supernatants were saved and used as the “detergent-soluble fraction.” The residual structure remaining in the pellet was washed with PBS, extracted with RIPA buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA) and used as the “detergent-resistant fraction.” Extracts from each step were analyzed by Western blots.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at [doi:10.1016/j.bbadis.2011.09.015](https://doi.org/10.1016/j.bbadis.2011.09.015).

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